TEXAS FORENSIC
SCIENCE COMMISSION
Justice Through Science

1700 North Congress Ave., Suite 445
Austin, Texas 78701

June 10, 2016

Chief Art Acevedo, Austin Police Department
715 E. 8th Street
Austin, TX 78701 and
Via email to Commander Nicholas Wright at
Nicholas.Wright@austintexas.gov

Ms. Rosemary Lehmberg, Travis County District Attorney
P.O. Box 1748
Austin, TX 78767 and
Via email to Assistant District Attorney Brandon Grunewald at
Brandon.Grunewald@traviscountytx.gov

Re: Recent Assessment of Austin Police Department Forensic DNA Laboratory

Dear Chief Acevedo and District Attorney Lehmberg:

The Texas Forensic Science Commission (Commission) in conjunction with the American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) conducted a site assessment of the Austin Police Department Crime Laboratory's DNA Section (APD DNA Lab) on May 27-28, 2016 and June 6, 2016. The review team consisted of Dr. Bruce Budowle, Director of the University of North Texas Center for Human Identification, D. Jody Koehler ASCLD/LAB Assessor and Manager of the DNA Section for the Texas Department of Public Safety Crime Laboratory in Austin, and Lynn Garcia, General Counsel for the Commission. The DNA analysis assessment was performed on May 27-28, 2016 (Budowle/Koehler/Garcia), and a review of the APD DNA Lab's forensic biology screening operations was conducted on June 6, 2016 (Koehler/Garcia).

The purpose of this letter is to communicate some initial observations that may affect stakeholders. The Commission will discuss the issues outlined below in further detail at its quarterly meeting on July 8, 2016. A comprehensive report will follow after deliberations at the meeting. The summary below highlights key issues in the interim. Observations are limited to the DNA section of the laboratory.

1. Establishment and Continuation of "Quant-Based" Stochastic Threshold

Beginning in 2010, the APD DNA Lab adopted a stochastic threshold (ST) using the quantity of DNA in a sample as a method for determining potential stochastic effects such as allele dropout, allele stacking/sharing, etc. This approach is referred to throughout this letter as the APD DNA Lab's "quant-based ST," and this approach was the primary catalyst for the site assessment. Using a quant-based ST to determine potential stochastic effects in DNA mixtures is neither scientifically valid nor supported by the forensic DNA community. The review team is aware of no peer-reviewed journal article citing the acceptance of a quant-based ST for mixture interpretation.

In adopting and continuing the use of a quant-based ST from 2010 to the present, the Technical Leaders (TLs) and senior analysts in the APD DNA Lab appear to have misunderstood language from the Scientific Working Group on DNA Analysis Methods (SWGDAM) Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories (*See* **Attachment A**) and from Dr. John M. Butler's textbook, "Advanced Topics in Forensic DNA Typing: Methodology" (*See* **Attachment B**) as supporting the use of a quant-based ST for assessing stochastic effects in DNA mixtures. Of greater concern, the analysts themselves were aware the quant-based ST was ineffective because they observed stochastic effects (e.g., allele dropout) in their casework *even when* the quantity of DNA in the sample exceeded the laboratory's own quant-based ST.

While analysis of DNA quantitation is one step in determining whether to proceed with Polymerase Chain Reaction (PCR) amplification at the outset of a case, the quantity of DNA is not an appropriate metric to assess potential stochastic effects that occur during amplification for DNA mixture evidence. An appropriate ST at the interpretation stage is based on the amount of the signal, measured in relative fluorescence units (RFUs), which is captured on the resultant electropherogram. While other laboratories both in and outside of Texas may not always have employed a dual threshold (analytical and stochastic), the Commission has identified no other laboratory in Texas, or elsewhere, that chose a quant-based ST after SWGDAM recommended in 2010 that an ST be incorporated as a tool for guarding against possible stochastic effects.

Moreover, a review of the APD DNA Lab's validation data used in support of the quant-based ST shows the study was lacking sufficient data to support selection of any ST. Only three samples were used with nine different dilutions (1.75-0.0029296 ng).[1] The dilutions were incorrectly prepared, with the individual transferring sample volumes of 0.005 uL of sample for amplification set-up, resulting in incorrect amounts of material being placed into reactions because the sample volume was too low to be accurately measured utilizing the tools available in the laboratory. These insufficiencies resulted in a quant-based ST validation study that was not supportable. The inadequate outcome was further demonstrated by subsequent analyst observations of stochastic effects even in cases where the quantity of DNA was higher than the very quant-based ST established as

[1] The team has concerns about whether the analysts performing the study understood the appropriate use of significant figures.

result of the validation study. Though analysts raised concerns about the quant-based ST with two consecutive TLs, the quant-based ST remained in effect.

2. Suspect or Victim-Driven Combined Probability of Inclusion (CPI)

Analysts typically conducted an initial review of evidentiary profiles before reviewing suspect or victim reference profiles. However, the analysts decided whether a locus would be used for statistical calculations depending upon the alleles observed in the known profile (whether suspect or victim). When analysts compared the evidence and known reference profiles, they assessed the comparison based on whether the suspect or victim was "dropping out" at a particular locus. If an allele was missing, they invoked allele dropout as a reason for not using the locus for statistical purposes. The appropriate approach is to *decide which locus (or loci) should be used first* based on whether there may be stochastic effects *as indicated by the overall analysis of the evidentiary sample*, not on which alleles are present or absent based on the victim or suspect known profiles.

3. Unclear Use of Protocol Deviation

In one case, the review team observed a deviation from protocol that did not appear to be supported by the documentation in the case file. The item in question had a quantity of DNA at 0.05025 ng amplified. This amount of DNA is lower than the quant-based ST established in the APD DNA Lab's standard operating procedures (SOP). (*See* **Attachment C**.) According to the SOP, for DNA quantities amplified below 0.0625 ng, the entire profile should be called uninterpretable if it is a mixture. When asked, the analyst explained the TL had signed a deviation allowing the profile to be interpreted as a major/minor mixture. While the written deviation did indeed permit the analyst to consider the profile as a major/minor mixture, it did not state the analyst could proceed and interpret the profile despite the SOP's clear guidance that a profile at 0.05025 ng amplified should be considered uninterpretable *even for major/minor mixtures.*

4. Contamination Events

In one case, the team observed potential carry-over contamination between the epithelial cell fraction from the victim's vaginal swab and the epithelial cell fraction from the penile swab of an individual subsequently determined to be unrelated to the offense. The analyst reported a three-person mixture and concluded the victim could not be excluded as a contributor to the epithelial cell fraction from the penile swab of this unrelated individual. When retested by another laboratory, the results of testing for the penile swab indicated a two-person mixture and excluded the victim, thus indicating possible contamination between samples during the original testing by the APD analyst. This case raises two important issues: (1) why the possible contamination between samples was not considered by the analyst or technical reviewer before the report was issued; and (2) whether analysts understand the role of the quality assurance process in addressing suspected contamination and assessing whether or not the contamination was an isolated event.

In addition, the review team observed 10 cases in which a reagent blank was contaminated. The reagent blank contained 8 peaks above the analytical threshold (75 RFUs). Peaks ranged in height from 103-744 RFUs. APD DNA Lab Staff traced the contamination back to the analyst's extraction reagents. Results from the 10 cases were reported, under the theory that because the alleles observed in the contaminated reagent were not observed in the evidentiary samples, the contamination in the reagent blank must not have affected the evidentiary samples. The APD DNA Lab's SOP allowed the TL to sign off on reporting the evidentiary samples despite significant reagent blank contamination without providing any defined criteria for when such signoff was appropriate. Clearly defined criteria in the SOP would remove the subjectivity in decision-making for contamination incidents involving reagent blanks.

5. Use of AP Reagent

The forensic biology screening analysts use a SERI Acid Phosphatase (AP) reagent beyond the "make fresh daily" instructions on the reagent bottle. APD DNA Lab analysts are instructed to make the AP reagent when needed, which could be anywhere from a few days to 2-4 weeks or until they run out of prepared reagent. Though analysts perform a quality check of the reagent daily, there is no supporting documentation on the criteria (e.g., time frame for development of color reaction and intensity of the color reaction) for assessing whether the AP reagent is performing as desired. In one study by the Ohio Bureau of Criminal Identification, loss of activity of the AP reagent was observed when exceeding the "make fresh daily" instructions. (*See* **Attachment D.**) Subjectivity in analysis and possible loss of strength in an AP reagent could lead analysts to miss potential semen stains when those stains are significantly weaker than the positive control. As provided in the FBI's Quality Assurance Guidelines (QAS), if chemical reagents are to be used beyond expiration dates (or in this case outside the manufacturer's instructions), such use should be supported by validation data. When asked to supply the validation data to support the extended use of the reagent, the APD DNA Lab was unable to do so.

6. The Role of Accreditation and SWGDAM

DNA laboratories are required to undergo external audits (individuals external to the lab conduct the audit) every other year. On the off years, they are required to perform an internal audit (individuals associated with the lab conduct the audit). ASCLD/LAB performed assessments at the laboratory and the quant-based ST was not questioned. Moreover, no deficiencies in validation studies were observed, though such problems were obvious. The depth of the DNA training program was also not questioned. The same lack of findings occurred with external QAS audits (not always associated with ASCLD/LAB) during the relevant time period. In 2010, the ASCLD/LAB assessor either did not review the ST validation study or did not appreciate that quantity of DNA was an inappropriate way to establish an ST for mixture interpretation. More than one analyst stated the quant-based ST was discussed with an auditor but it is unclear with which auditor this discussion occurred. In 2015, an ASCLD/LAB auditor reviewed the Fusion 30 cycle validation data but no findings were made even though there were deficiencies

in that validation as well. After the quant-based ST was established in 2010, there does not appear to have been another external review of the ST study until the one conducted by this review team.

These observations raise legitimate questions regarding the limits of accreditation and the consistency of assessor teams. Specifically: (a) Are the scope and limitations of accreditation well understood by the criminal justice community? (b) Do assessors consistently consider whether a laboratory's protocols and underlying validation are based on sound scientific principles or do they limit their review solely to determining whether the laboratory has a protocol in place that it follows? (c) Should assessors re-review validation data from prior years considering that validation studies are relied upon to build subsequent protocols?

The forensic DNA community also relies heavily on SWGDAM for guidance on how to best address complex issues that arise concerning mixture interpretation and many other issues. Though SWGDAM guidelines provide a tremendous amount of necessary and helpful information to the community, it would be a mistake to believe either the organization as a whole or its individual members view their role as intervening in the protocol decisions and practices of individual forensic DNA laboratories. Thus, though SWGDAM is a useful resource, it is not an oversight or standard-setting body. Until the National Institute of Standards and Technology (NIST) Organization of Scientific Area Committees (OSAC) publishes standards in DNA analysis that are well understood and implemented by the community, oversight and standard development for forensic DNA laboratories will remain completely within the umbrella of the accreditation bodies, the FBI's QAS and state-level forensic commissions to the extent they exist.

We understand APD DNA Lab management is working with ASCLD/LAB to amend the scope of its accreditation to temporarily suspend forensic DNA analysis, including forensic biology screening, which will allow the APD DNA Lab the necessary time to address observations as well as to re-train and re-qualify its analysts to acceptable standards. We also understand the APD DNA Lab is in the process of arranging for a technical expert to spearhead this comprehensive program. This proactive approach should allow the APD DNA Lab to emerge as a stronger forensic DNA laboratory in the long-term.

Commissioners may offer additional observations and recommendations at the quarterly meeting on July 8, 2016. If you have any questions, please do not hesitate to contact us.

Sincerely,

Lynn Garcia
General Counsel

Vincent J.M. Di Maio, MD
Presiding Officer

ATTACHMENT A

3. Interpretation of DNA Typing Results

3.1. Non-Allelic Peaks

Because forensic DNA typing characterizes STR loci using PCR and electrophoretic technologies, some data that result from this analytical scheme may not represent actual alleles that originate in the sample. It is therefore necessary, before the STR typing results can be used for comparison purposes, to identify any potential non-allelic peaks. Non-allelic peaks may be PCR products (e.g., stutter, non-template dependent nucleotide addition, and non-specific amplification product), analytical artifacts (e.g., spikes and raised baseline), instrumental limitations (e.g., incomplete spectral separation resulting in pull-up or bleed-through), or may be introduced into the process (e.g., disassociated primer dye). Generally, non-allelic data such as stutter, non-template dependent nucleotide addition, disassociated dye, and incomplete spectral separation are reproducible; spikes and raised baseline are generally non-reproducible.

3.1.1. The laboratory establishes criteria based on empirical data (obtained internally or externally), and specific to the amplification and detection systems used, to address the interpretation of non-allelic peaks. The guidelines address identification of non-allelic peaks and the uniform application, across all loci of a DNA profile, of the criteria used to identify non-allelic peaks.

3.1.1.1. In general, the empirical criteria are based on qualitative and/or quantitative characteristics of peaks. As an example, dye artifacts and spikes may be distinguished from allelic peaks based on morphology and/or reproducibility. Stutter and non-template dependent nucleotide addition peaks may be characterized based on size relative to an allelic peak and amplitude.

3.1.1.2. While the application of an analytical threshold may serve to filter out some non-allelic peaks, the analytical threshold should be established based on signal-to-noise considerations (i.e., distinguishing potential allelic peaks from background). The analytical threshold should not be established for purposes of avoiding artifact labeling as such may result in the potential loss of allelic data.

3.1.1.3. The laboratory establishes guidelines addressing off-scale data. Fluorescence detection instruments have a limited linear range of detection, and signal saturation can result in off-scale peaks. Following peak detection, such peaks in the analyzed data are assigned an artificial height value which is not representative of the true amplitude. Peak

height values for off-scale peaks should not be used in quantitative aspects of interpretation (e.g., stutter and peak height ratio assessments).

3.2. Application of Peak Height Thresholds to Allelic Peaks

Amplification of low-level DNA samples may be subject to stochastic effects, where two alleles at a heterozygous locus exhibit considerably different peak heights (i.e., peak height ratio generally <60%) or an allele fails to amplify to a detectable level (i.e., allelic dropout). Stochastic effects within an amplification may affect one or more loci irrespective of allele size. Such low-level samples exhibit peak heights within a given range which is dependent on quantitation system, amplification kit and detection instrumentation. A threshold value can be applied to alert the DNA analyst that all of the DNA typing information may not have been detected for a given sample. This threshold, referred to as a stochastic threshold, is defined as the value above which it is reasonable to assume that allelic dropout has not occurred within a single-source sample. The application of a stochastic threshold to the interpretation of mixtures should take into account the additive effects of potential allele sharing.

3.2.1. The laboratory establishes a stochastic threshold based on empirical data derived within the laboratory and specific to the quantitation and amplification systems (e.g., kits) and the detection instrumentation used. It is noted that a stochastic threshold may be established by assessing peak height ratios across multiple loci in dilution series of DNA amplified in replicate. The RFU value above which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele has not occurred constitutes a stochastic threshold.

3.2.1.1. If measures are used to enhance detection sensitivity (i.e., allelic height), the laboratory should perform additional studies to establish independent criteria for application of a separate stochastic threshold(s). Such measures may include but not be limited to increased amplification cycle number, increased injection time, and post-amplification purification/concentration of amplified products.

3.2.1.2. For samples for which an assumption can be made as to the number of contributors, the laboratory should establish criteria for comparison of allelic peaks which fall below the stochastic threshold. As an example, if a locus in an assumed single-source sample exhibits two peaks, one or both of which are below the stochastic threshold, the laboratory may use that locus for comparison purposes. Also, the presence of male DNA may be established based on a Y-allele at amelogenin that is below the stochastic threshold.

3.2.2. If a stochastic threshold based on peak height is not used in the evaluation of DNA typing results, the laboratory must establish alternative

criteria (e.g., quantitation values or use of a probabilistic genotype approach) for addressing potential stochastic amplification. The criteria must be supported by empirical data and internal validation and must be documented in the standard operating procedures.

3.3. Peak Height Ratio

Intra-locus peak height ratios (PHR) are calculated for a given locus by dividing the peak height of an allele with a lower RFU value by the peak height of an allele with a higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage.

3.3.1. The laboratory should establish PHR requirements based on empirical data for interpretation of DNA typing results from single-source samples. Different PHR expectations can be applied to individual loci (e.g., 70% for D3S1358, 65% for vWA, etc.); alternatively, a single PHR expectation can be applied to multiple loci (e.g., 60%).

3.3.1.1. The laboratory may evaluate PHRs at various DNA template levels (e.g., dilution series of DNA). It is noted that different PHR expectations at different peak height ranges may be established.

3.3.2. PHR requirements are only applicable to allelic peaks that meet or exceed the stochastic threshold.

3.4. Number of Contributors to a DNA Profile

Generally, a sample is considered to have originated from a single individual if one or two alleles are present at all loci for which typing results were obtained (although tri-allelic loci may occur), and the peak height ratios for all heterozygous loci are within the empirically determined values. It is noted that peak height imbalances may be seen in the typing results from, for example, a primer binding site variant that results in attenuated amplification of one allele of a heterozygous pair.

A sample is generally considered to have originated from more than one individual if three or more alleles are present at one or more loci (excepting tri-allelic loci) and/or the peak height ratios between a single pair of allelic peaks for one or more loci are below the empirically determined heterozygous peak height ratio expectation. Generally, the minimum number of contributors to a mixed sample can be determined based on the locus that exhibits the greatest number of allelic peaks. As an example, if at most five alleles are detected per locus, then the DNA typing results are consistent with having arisen from at least three individuals.

ATTACHMENT B

Sampling Limitations with Sperm Cells

New sampling techniques such as laser capture microdissection (see Chapter 2) enable collection of specific cells. Keep in mind that individual sperm cells contain only half of the genomic material from the donor. Therefore, multiple sperm cells will need to be collected in order to represent the complete DNA profile (D.N.A. Box 11.5).

Whole Genome Amplification

A DNA enrichment technology known as whole genome amplification (WGA) has been explored as a possible method for recovery of limited quantities of DNA from evidentiary samples (Bergen et al. 2005, Hanson & Ballantyne 2005, Ballantyne et al. 2007). WGA involves a different DNA polymerase (phi29) than the TaqGold enzyme commonly used in forensic DNA analysis and amplifies the entire genome using random hexamers as priming points. The WGA enzymes work by multiple displacement amplification (MDA), which is sometimes referred to as rolling circle amplification. MDA is isothermal with an incubation temperature of 30°C and requires no heating and cooling like PCR.

QIAGEN (Valencia, CA) and Sigma-Aldrich (St. Louis, MO) both offer phi29 DNA polymerase cocktails for performing WGA. The kit sold by QIAGEN is called REPLI-g while Sigma-Aldrich's kit is GenomePlex. Yields of 4 μg to 7 μg of amplified genomic DNA are possible from as little as 1 ng of starting material. The phi29 enzyme has a high processivity and can amplify fragments of up to 100 kb because it displaces downstream product strands enabling multiple concurrent and overlapping rounds of amplification. In addition, phi29 has a higher replication fidelity compared to Taq polymerase due to 3′–5′ proofreading activity.

While all of these characteristics make WGA seem like a possible solution to the forensic problem of limited DNA starting material, studies have found that stochastic effects at low levels of DNA template prevent WGA from working reliably (Schneider et al. 2004). Allele drop-outs from STR loci were observed at 50 pg and 5 pg levels of starting material (Schneider et al. 2004) just as are seen with current LT-DNA testing. Work with "molecular crowding" materials such as polyethylene glycol, where the amount of DNA is enriched in localized areas of a sample, has shown improved success with STR typing from low amounts of DNA (Ballantyne et al. 2006).

It is possible that WGA may play a limited role in enriching samples for archiving purposes that are in the low ng range (Lasken & Egholm 2003), but it will probably not be the end-all solution to LT-DNA samples in the low picogram range. Thus, it appears, as with regular PCR techniques, stochastic selection of alleles present in low-level DNA samples limits the effectiveness of WGA to several hundred picograms in order to recover a full profile with a single amplification attempt.

Caution in Relying on DNA Quantitation Values

Is it possible to ascertain that sufficient DNA material exists to obtain reliable results where the DNA profile obtained is expected to accurately reflect the source of the biological sample? There are two primary points in the DNA testing process where potential DNA reliability may be assessed: (1) at the DNA quantitation stage prior to performing PCR

amplification of the STR markers of interest or (2) by examining the peak heights—and peak height ratios in heterozygous loci—in the STR profile obtained.

An empirically determined threshold (usually termed a "stochastic threshold") may be used at either the DNA quantitation or data interpretation stage to assess samples in the potential danger zone of unreliable results. For example, if the total amount of measured DNA is below 150 pg, a laboratory may decide not to proceed with PCR amplification assuming that allelic drop-out due to stochastic effects is a very real possibility. Alternatively, a laboratory may proceed with testing a low-level DNA sample and then evaluate the peak height signals and peak ratios at heterozygous loci. When peak height ratios for heterozygous loci in single-source samples dip below 60%, there is an indication that stochastic effects are significant which would make it challenging to reliably pair alleles into major and minor genotypes with mixtures. This topic will be covered more extensively in the forthcoming volume *Advanced Topics in Forensic DNA Typing: Interpretation*.

Since the advent of quantitative PCR (qPCR) assays, DNA quantitation tests have become more sensitive—enabling quantities as small as a few genomic copies to be detected (see Chapter 3). However, it is important to keep in mind that qPCR is also subject to stochastic variation especially on the low end of DNA quantity measurement. Thus, numbers in the low picogram range may not be reliable and results with little or no "detectable" DNA may still amplify with STR kits (Cupples et al. 2009; see also D.N.A. Box 3.3).

In an early paper discussing stochastic effects and the limitations of PCR assays, Walsh et al. (1992) proposed avoiding stochastic effects by adjusting the number of PCR cycles in an assay so that the sensitivity limit is around 20 or more copies of target DNA. In other words, their goal was to enable a full DNA profile to be reliably obtained with approximately 125 pg of DNA. Below roughly that amount, allele and locus drop-out would be expected and partial DNA profiles would result (Walsh et al. 1992). Obtaining a partial DNA profile is an indication that a low-level DNA amplification has occurred.

Depending on the STR typing kit primer and DNA polymerase concentrations and the fluorescent dye sensitivities, the number of PCR cycles is typically set by manufacturers in the range of 28 cycles to 32 cycles. However, as noted previously, STR kits certainly work beyond manufacturer recommended cycle numbers. If laboratories choose to increase cycle numbers beyond what is recommended by manufacturers, validation studies are needed to help set appropriate interpretation guidelines.

Thresholds Are Difficult to Set with Enhanced Detection Methods

Stochastic thresholds, such as 150 RFU or even 500 RFU, may not apply for enhanced detection methods that include increasing the number of PCR cycles. Instead, independent replicate amplifications and concensus profile development are necessary to compensate for allele drop-out and drop-in. Computer software that involves probabilistic modeling to data, such as LoComatioN (Gill et al. 2007), will be important to future advances with low-level DNA analysis.

STRBase Website on Low Template DNA

A low template DNA section of the NIST STRBase website was launched in October 2009 following the International Symposium on Human Identification LCN Panel. This website,

ATTACHMENT C

of the profile should be considered when determining whether a profile is a partial profile or not, and all individual locus interpretations must occur prior to comparing to the known reference samples in the case. Some profiles may contain too many contributors, or be of poor quality, to allow the profile to be used for interpretation. The profile should be designated as inconclusive and the analyst's reason for doing so shall be documented in the case record. This determination shall be agreed to by the technical reviewer and, if necessary in the case of dispute, agreed to by the technical leader. See below for more guidance on interpreting and reporting partial profiles.

Stochastic effects

Decreasing levels of template DNA may lead to stochastic effects which may under-represent one of the alleles in a locus. Using a minimum analytical threshold of 75 RFU, the following guidelines will be followed for interpreting data from low concentration samples:

Concentration	Single Source	Mixture with Major Component	Mixture with no Major Component
>0.3 ng	X	X	X
Between 0.0625 ng and 0.3 ng	X	Interpret loci from the major profile that contain heterozygous loci. The minor profile will be deemed uninterpretable.	The entire profile is uninterpretable
<0.0625 ng	May interpret heterozygous loci (>75 RFU) or designate entire profile as uninterpretable	The entire profile is uninterpretable	The entire profile is uninterpretable

NOTE: X indicates that this combination of criteria does not meet the minimum criteria for stochastic amplification and the special guidelines for stochastic amplification are not applicable. Interpret according to the standard interpretation guidelines.

The table above represents commonly encountered general guidelines. If a departure from the above guidelines is determined to be necessary after discussion between the analyst and technical reviewer, approval from the technical leader is necessary prior to issuance of a test report.

Mixtures

Samples from crime scene evidence may contain DNA from more than one individual. The entire profile should be used to determine if there is sufficient information to conclude that the sample contains DNA from more than one person. The analyst should be aware that mixtures can consist of full and/or partial profiles from multiple individuals, and a full profile from each component is not assumed due to potential dropout,

ATTACHMENT D

Forensic Detection of Semen I. The Acid Phosphatase Test

Dale L. Laux, M.S.

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Introduction

Acid phosphatase is an enzyme secreted by the prostate gland that is present in large amounts in seminal fluid. It, like psa (prostatic specific antigen), is not unique to the prostate and can be found in other biological fluids including vaginal secretions. It is therefore considered a presumptive chemical test for the presence of semen and semen must be confirmed by other means (sperm detection or psa detection using membrane test systems).

Testing for the presence of acid phosphatase can be extremely helpful however, in locating semen stains on clothing and for testing swabs from sexual assault cases. A strong positive reaction generally indicates that semen is present and that further testing is warranted.

For an excellent review on the history of acid phosphatase detection, see Gaensslen [1]. A number of testing methods exist for the sampling of items for the presence of acid phosphatase. The enzymatic breakdown of sodium-α-naphthyl phosphate by acid phosphatase and the subsequent conversion of o-dianisidine to a colored compound by the free naphthyl is a recognized test procedure for the detection of semen [2]. The Serological Research Institute (SERI) produces a powder they call ap spot test. When the powder is reconstituted in water, it can be used to screen stains and swabs for the presence of semen.

The sensitivity and stability of the product are discussed.

Materials and Methods

Sensitivity

Acid phosphatase was obtained from Sigma Chemical Company. The product number was P-1146, Lot 051K7038 and was isolated from potato. 50 units were purchased, consisting of 7.5 mg of solid having an activity of 6.7 units/mg (50.25 units). The solid was dissolved in 200 µL of deionized water. 100 µL of this solution (25 units) was added to a cotton-tipped swab that was allowed to air dry. 50 µL deionized water was added to the remaining 100 µL, mixed and 100 µL of this solution (17 units) was added to a

cotton-tipped swab that was allowed to air dry. Subsequent dilutions were made in this manner resulting in dry cotton-tipped swabs having the following units of acid phosphatase: 25, 17, 5.6, 1.8, 0.6, 0.2, 0.05 and 0.02.

Testing of these dry swabs was conducted in the following manner. Deionized water was added to a small piece of Whatman filter paper #3. Each swab was pressed against the filter paper strongly between thumb and forefinger for ten seconds. A single drop of freshly prepared SERI ap spot test (Lot 1562) was added to each piece of filter paper and color changes were recorded after 10 minutes.

Stability

SERI ap spot test (Lot 1562) was prepared fresh daily and used for case analysis. The reagent was maintained in a small glass dropper bottle protected from light with tape at room temperature. At the end of the business day (approximately 8 hours), the reagent was placed in a plastic15 mL Falcon tube and refrigerated. The following morning, fresh ap spot test was prepared and kept on the lab bench along with the previous preparation. This procedure was followed for the three remaining days of the week.

Whatman #3 filter paper was moistened and a cotton-tipped swab containing 25 units of acid phosphatase was pressed to 5 areas of the paper (following the procedure described previously). The same procedure was followed with 17 units of acid phosphatase.

SERI ap spot test reagent (fresh, 1 day, 2 days, 3 days, 4 days and 5 days old) was added to the filter paper and color changes were recorded after 10 minutes.

The same methods were followed using SERI ap spot test reagents that were stored frozen for 1 to 5 days; however, the reagents were not removed from the freezer daily.

Results and Discussion

Sexual assault kits and clothing are routinely submitted to crime laboratories for examination for the presence of semen. Typically, forensic scientists conduct visual examinations for stains followed by examination with an alternate light source on clothing and bedding items. This is generally followed by testing of stains for the presence of acid phosphatase, an enzyme secreted by the prostate and found in high levels in semen. Swabs collected from sexual assault survivors are generally tested for the presence of acid phosphatase followed by tests for the presence of spermatozoa, and P30 if necessary.

It is customary to test stained areas and swabs collected from the survivor indirectly. In other words, a transfer method involving wet or dry cotton-tipped swabs or moistened filter paper applied as an overlay to a stained area or swabbing is employed. As recommended by Barnett, et.al. [3], presumptive test reagents should NEVER be applied directly to items of evidence.

Following this methodology, experiments were designed to determine the sensitivity of one acid phosphatase test. The Serological Research Institute (SERI) sells a product they call ap spot test. It contains sodium-α-naphthyl phosphate and o-dianisidine (Fast Blue B). If acid phosphatase is present in a sample and a drop of the ap spot test is added, the enzyme catalyzes the breakdown of sodium-α-naphthyl phosphate producing free naphthyl that reacts with o-dianisidine producing a purple colored compound.

Results of Sensitivity Tests

Freshly prepared ap spot test gave positive results with acid phosphatase diluted to 0.18 to 0.6 units.* A photograph of the results appears in Figure 1.

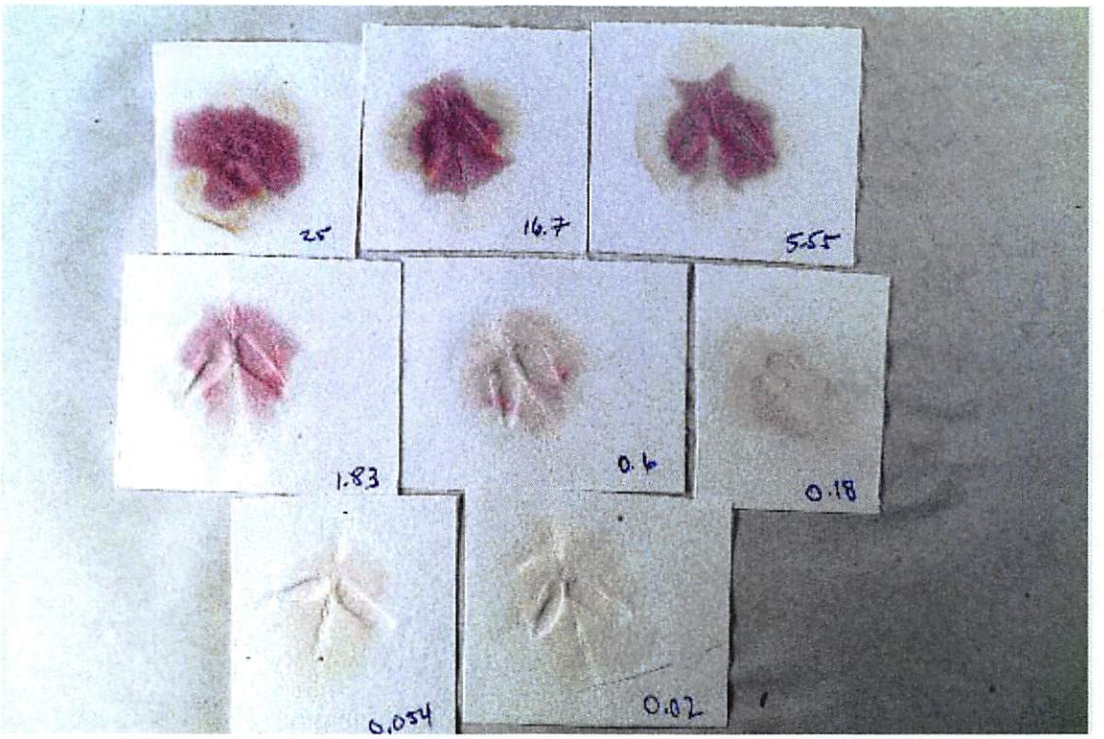

Figure 1. One drop of ap spot test added to moistened filter paper containing diluted acid phosphatase (25 to 0.02 units). Positive reaction (purple color change) obtained at 0.6 units.

Sensabaugh [4] published results of experiments designed to quantitate the levels of endogenous and postcoital vaginal acid phosphatase. He standardized data from several investigators including his data and obtained a range of endogenous vaginal acid phosphatase of 0.023 to 4.902 units. SERI's ap spot test would certainly react with these endogenous levels of acid phosphatase. Hence the presumptive nature of the ap test and the requirement that the presence of semen be confirmed in another manner.

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*One unit will hydrolyze 1.0 μmole of p-nitrophenyl phosphate per minute at pH 4.8 at 37 °C

Results of Stability Tests

The directions supplied with SERI's ap spot test state to prepare the reagent daily. The Fast Blue B dye is light sensitive. At room temperature, on the lab bench and in the light, the ap spot test will begin to turn yellow and brown material will precipitate out.

The results of the stability experiments are shown in Figure 2.

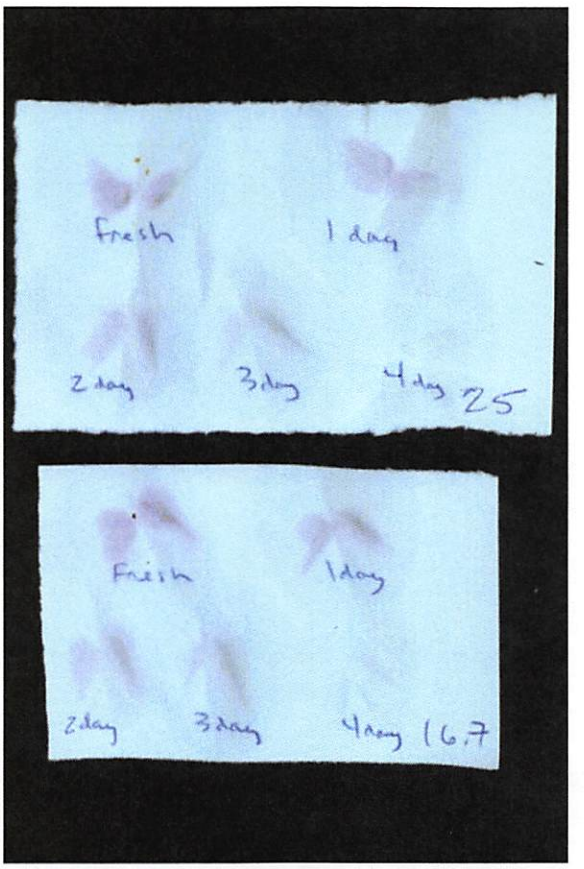

Figure 2. Photograph of filter paper with 25 and 12 units of acid phosphatase and drops of fresh, 1 day, 2 day, 3 day and 4 day old ap spot test reagent.

A decrease in activity was observed in 1 day old ap spot test reagent, however it still reacted fairly well. By two days, the activity of the reagent dropped significantly and by four days, the reagent has lost the ability to detect 25 units of acid phosphatase.

Tests were conducted to determine whether freezing the reagent could enhance stability. The results of this experiment are shown in Figure 3. As can be seen in Figure 3, freezing did enhance the stability of the reagent. However, the frozen reagents were not

removed from the freezer and thawed on the laboratory bench daily, but remained in the freezer.

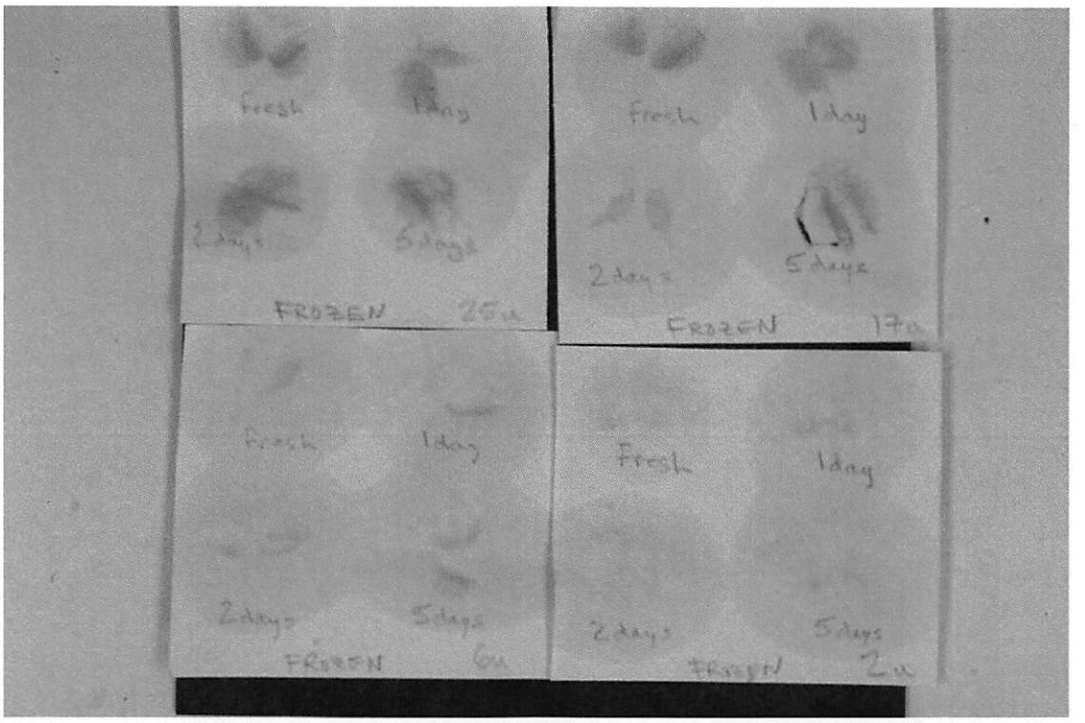

Figure 3. Varying units of acid phosphatase (25 u, 17 u, 6u and 2 u) deposited on filter paper to which ap spot test, frozen for various days (fresh, 1 day, 2 days, 5 days), was added.

The ap spot test stored frozen for 5 days worked as well as the freshly prepared reagent. However, once thawed and left on the lab bench, this reagent would degrade just as fresh or refrigerated reagent.

Interpretation of the color change indicating a positive result can be subjective. As seen in Figure 1, a deep, dark purple color change, especially if it occurs rapidly, strongly indicates the presence of semen and would demand further testing. Light results such as 0.6 to 0.18 units (Figure 1) may be the result of very weak semen stains or endogenous acid phosphatase levels.

Occasionally, color changes having a tannish hue are found on swabs taken from survivors, especially rectal swabs. Figure 4 shows one such result. This is a typical result from rectal and anal swabs and should not be confused with a positive AP reaction.

Subsequent testing for P30 and spermatozoa was negative. Certainly, these results can't be ignored, but the experienced analyst will recognize these as negative results, and not a true purple color change indicating the presence of semen.

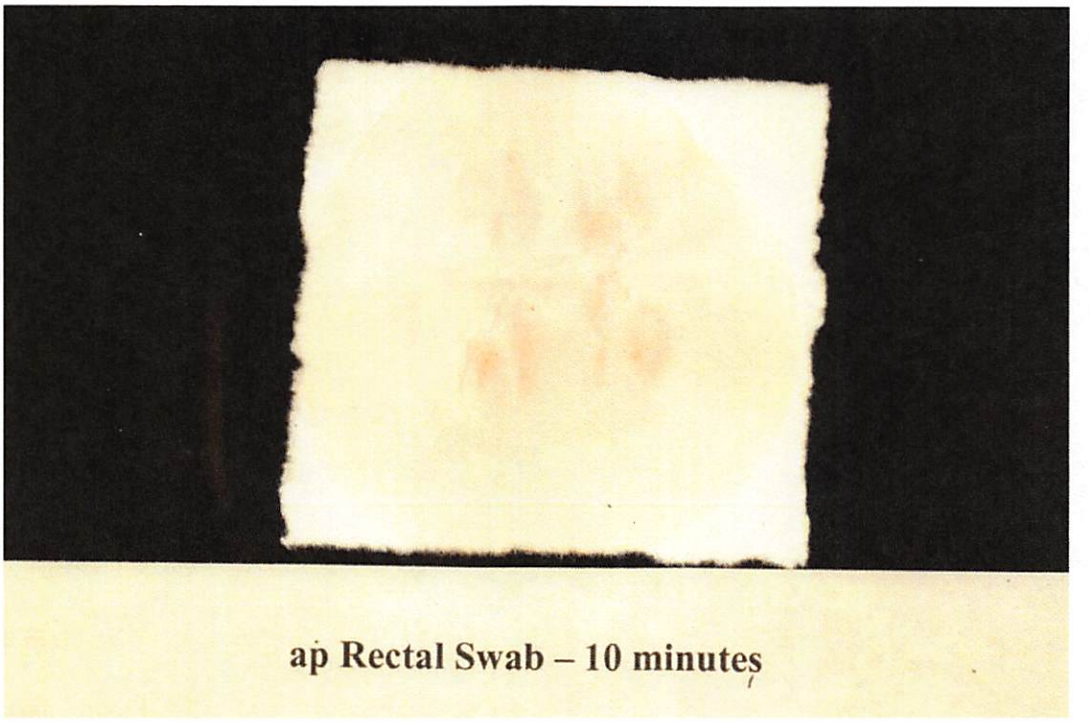

Figure 4. Typical results obtained from ap spot test added to filter paper transfer from a rectal swab. No semen was present on the swab.

Experience cannot be overemphasized and senior forensic biologists should make a habit of passing on their knowledge to new analysts. Schiff [5] stated "after 14 year's (sp) experience with the AP test, the author has found it to have great merit as a test for the identification of seminal fluid in the absence of spermatozoa". He continues, "because he has used the test qualitatively rather than quantitatively, he has established no arbitrary, numerical cutoff as to when the test is to be declared positive and when negative". He states that the test "is only as dependable as the physician, chemist, or pathologist who performs it".

Schiff lists three guidelines that he maintains should be followed in conducting the AP test:

1. The reagents must be freshly prepared.

   He found that the diazo-coupling agent that originally was clear and lightly tinted began to precipitate after 12 hours. This author has experienced the same result and recommends that it be prepared fresh daily.

2. The examiner must follow the same protocol in every case.

   In other words, press a swab to a piece of filter paper for the same time period applying the same pressure each and every time. Add the same number of drops of AP spot test and wait the same length of time each and every time. Developing consistency in the conducting the test will make the analyst more comfortable in interpreting the results.

3. The examiner must not deviate from his/her method of reading the test.

   After the analyst gains confidence in conducting the test, and performs a sufficient number of confirmatory tests on various test results, the analyst will come to realize what is a true positive reaction.

Conclusion

Testing for acid phosphatase remains a valuable presumptive test for the screening of swabs collected from sexual assault survivors and for the testing of stains found on clothing and bedding. The experienced forensic biologist knows that all stains that fluoresce are not necessarily semen and all semen stains do not fluoresce. In addition, semen is a heterogeneous fluid and portions of a deposited stain will contain various levels of acid phosphatase, P30 and spermatozoa. Examination of a pair of panties with an alternate light source and extraction of all the stains that fluoresce followed by psa analysis may yield semen, however, it may not, and it does not appear to this author to be the best use of time and expenses. Acid phosphatase mapping is an inexpensive and quick method for screening such stains.

Years ago, forensic biologists (serologists) were taught what was termed "a systematic approach to the analysis of semen evidence" developed by Blake, Sensabaugh and Bashinski [6]. The three major steps consisted of locating the stain, estimating the amount of semen found and genetic analysis of the stain. With the advent of DNA, it seems possible that one could just cut a stain from a pair of underwear, extract it and generate a DNA profile. Obtaining the subject's DNA profile on the underwear, where it shouldn't be, should be conclusive proof of guilt. And perhaps it is. However, this analyst, trained in the "old school" feels that a more thorough analysis is warranted. Acid phosphatase mapping in locating stains and sperm quantitation of positive stains are important steps that can only aid the DNA analyst in interpreting the results.

It behooves the forensic biologist to utilize all of the methods available for optimum semen detection.

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